DNA Methylation and Primary Immune Thrombocytopenia

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DNA methylation is a heritable, stable, and also reversible way of DNA modification; it can regulate gene expression without changing the nucleotide sequences. Because it takes part in regulation of immune responses, the loss of methylation homeostasis in immune cells will result in autoimmune disease by inducing aberrant gene expression. Primary immune thrombocytopenia (ITP) is an acquired autoimmune disease with many immune deficiencies. Recently, it was well documented that abnormal DNA methylation is also involved in the etiology of ITP. In this review, we elucidate the role of DNA methylation in autoimmune diseases by summarizing the DNA methylation-sensitive genes and the relationship between DNA methylation and ITP.
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The autoimmune diseases are a series of disorders with various and complex defects in the immune system; the etiologies for some of them remain unclear today. Although DNA sequence changes have been claimed to be associated with susceptibility to disease onset, the different concordance rate of autoimmune diseases between monozygotic twins indicates that environment also participates in the development of autoimmunity.[1–4] These environmental factors effect immunity mainly by altering epigenetic regulation, which ultimately results in changes in gene expression.[5,6] DNA methylation is an epigenetic process that refers to heritable chromatin-based mechanisms in the regulation of gene expression without DNA alternation. This modification of DNA is time- and cell-specific, and thus it plays an important role in the regulation of many pathology processes, such as embryonic development, X chromosome inactivation, genomic imprinting, and cellular differentiation.[7–10]

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DNA METHYLATION

DNA methylation is mediated by DNA methyltransferases (DNMTs) through the addition of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine residues in CG dinucleotides.[11] There are five members in DNMT group: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. The function of DNMT1 is to methylate the hemimethylated sites generated during DNA replication. As the maintenance DNMT, it is implicated in stabilizing DNA methylation patterns during cell division.[12] DNMT3A and 3B are known as de novo DNA methyltransferases; they are responsible for the establishment of DNA methylation patterns during fetal development. In addition, DNMT3A and DNMT3B also exhibit non-overlapping functions in this procedure, with DNMT3B being specifically required for methylation of centromeric minor satellite repeats.[13,14] DNMT2 is not essential for global de novo or maintenance methylation of DNA because of its weak DNA methyltransferase activity; instead, it acts on methylation of small RNA.[15–17] DNMT3L is crucial for the establishment of maternal genomic imprints but lacks key methyltransferase motifs; it is more likely to act as a regulator of methylation by recruiting histone deacetylase (HADC) and stimulating de novo methylation by DNMT3A.[18–20]

DNA methylation is a stable but reversible epigenetic modification; thus DNA demethylation, which contributes to changes in the DNA methylation pattern, is also vital for biological processes. DNA demethylation includes passive and active mechanisms. Passive mechanisms require DNA replication

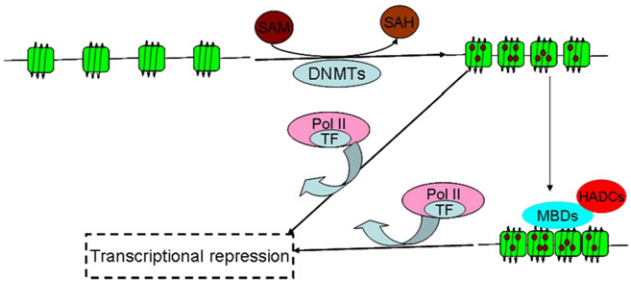

Figure 1. DNA methylation can repress gene transcriptions. DNA methylation is mediated by DNA methyltransferases (DNMTs) through the addition of a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine residues in CG dinucleotide. On the one hand, these methyl groups can inhibit gene expression directly by blocking transcription complex binding to DNA. On the other hand, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBDs can further recruit histone deacetylases (HADC), that eventually alter chromatin structures and lead to inaccessible transcription. Red hexagon stands for methyl group. Pol II: RNA polymerase II; TF: transcription factors.

by inhibition of DNMTs.[21] The active process is independent of DNA replication; it occurs by removing the methyl group by demethyltransferases, such as methyl-CpG-binding domain (MBD) proteins.[22]

DNA methylation can repress transcription through several mechanisms (Figure 1). First, methyl groups inhibit gene expression directly by blocking transcription complex binding to DNA.[23] Second, MBDs (MBD1, MBD2, MBD3, MBD4, and MECP2) are attracted to the methylated CpG dinucleotide. Since the binding of these proteins on methylated DNA also prevented the binding of transcription complex, MBDs were reported to be transcriptional repressors as well as DNA demethylases. In addition, MBDs can recruit HADC, eventually altering chromatin structures and leading to inaccessible transcription.[24,25]

The DNA methylated status of a genome is influenced by several factors. First, the 5-methylcytosine content in DNA declines with age, suggesting that the methylated level is related to the proliferative potential of organs.[26] Meanwhile, the DNA methylated level is different between males and females. As women have two X chromosomes, a high level of DNA methylation is used to inactivate one X chromosome. This X-inactivation results in women having equivalent X chromosome gene products as males.[9] In addition, food, drug, and environmental factors also impact DNA methylation.[5,6,27,28]

DNA METHYLATION AND AUTOIMMUNITY

Normal T cells treated with the DNA methylation inhibitor 5-azacytidine induce autologous B-cell differentiation and T-cell proliferation, and ultimately initiate autoimmunity.[29,30] Thus, DNA methyltransferase inhibitors, such as procainamide and 5-azacytidine, are sufficient to induce a lupus-like disease.[31] In addition, the lymphocytes of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) manifest global DNA hypomethylated status due to the abnormal expressions of DNMTs and MBDs,[32] which further demonstrates that DNA methylation plays a key role in autoimmune diseases.

What is the reason for hypomethylation in some autoimmune diseases? There is evidence that the impaired ERK and ras-MAPK pathway signaling in T cells results in decreased expression of DNMT1 and DNA hypomethylation.[33,34] This defective methylation capacity finally generates autoimmunity mainly through inducing overexpression of methylation-sensitive genes that are engaged in autoreactive immune responses in lymphocyte, including *IL-4, IL-6, CD70,* and *FoxP3* among others (Table 1).

CD11a

Lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) is a leukocyte cell surface heterodimer. It promotes intercellular adhesion by binding to members of the intercellular adhesion molecule family in immunological and inflammatory reactions.[35] The expression of LFA-1 is tissue-specific through the regulation of methylation and chromatin structure.[36] Normal T cells treated with DNA methylation inhibitors had increased CD11a expression directly for demethylation of the CD11a promoter.[37,38] Lu et al. found that the specific sequence flanking the CD11a promoter was hypomethylation in T cells from patients with active lupus or in normal T cells treated with 5-azacytidine and procainamide. Patch methylation of this region can suppress CD11a promoter function, which illustrated that altered methylation of specific genes may be relevant to the pathogenesis of lupus.[39]

CD70

CD70 is a costimulatory molecule of the tumor necrosis factor super family. It is expressed on activated dendritic cells, B cells, and T cells. CD27, the CD70 receptor, is also expressed on activated immunocytes. CD70–CD27 signaling provides a costimulatory signal for T-cell activation and survival. CD70 is another methylation-sensitive gene that is overexpressed in normal T cells treated with DNA methylation inhibitors, and these overexpressed CD70 contribute to overstimulated B cells and increased IgG production.[40,41] In SLE patients, CD70 expression is significantly elevated and

Table 1. Summary of DNA Methylation Involved in Autoimmune Diseases

Disease	Global DNA Methylation Levels	Methylation-Associated Enzymes	Methylation-Sensitive Genes		
			Genes	Methylation Status	Expression
SLE	Hypomethylation[79,80]	DNMT1↓[79]	*CD70*,[42] *CD40*,[73] *IL-4* and *IL-6*,[52] *IL-13*,[83]		
		DNMT3A, 3B↓[81]	*IL-2*,[69] *perforin*,[60] *CD11a*,[39] *CD5*,[77]	Hypomethylation	Increase
		MBD2, MeCP ↑[82]	*protein phosphatase 2*,[85], *IL-10* and *IL-1 recepor 2*,[32] *5-hydroxytryptamine receptor 1A*,[84] others[86–89]		
RA	Hypomethylation[90]	DNMT1↑	*CD40L*,[71] *IL-6*,[63] *CXCL12*,[91] *IL-1 recepor 2*[32]	Hypomethylation	Increase
		MBD2 ↑[90]	*IL-10*[32,92]	Inconsistent	Decrease
			Death receptor-3[93]	Hypermethylation	Decrease
Psoriasis	Inconsistent[94,95]	DNMT1↑	*p15* and *p21*,[96] *protein tyrosine phosphatase-1*[98]	Hypomethylation	Increase
		NBD2, MeCP↓[95]	*p14*[95]	Hypermethylation	Decrease
			p16[97,99]	Inconsistent	
MS	Hypomethylation[100]		*Protein tyrosine phosphatase-1*[101]	Hypermethylation	Decrease
			Peptidyl argininedeiminase 2[100,102]	Hypomethylation	Increase
SS	Hypomethylation[72,79,103]	DNMT1↓ MBD3, MBD4 ↓[79]	*CD70*,[44,99] *CD40L*[72]	Hypomethylation	Increase
T1D	Hypermethylated[104]	DNMT3B↑[56]	*Insulin*,[104] *FoxP3*[56]	Hypermethylation	Decrease
UC	Hypermethylated	DNMT1, DNMT3B↑[105]	*Multidrug resistance 1*,[106] *p16*,[107] *p14*,[108] *estrogen receptor-1*, *tumor suppressor candidate-3*,[109] others[105]	Hypermethylation	Decrease
			Protease-activated receptor 2[110]	Hypomethylation	Increase

Abbreviations: SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; MS, multiple sclerosis; SS, systemic sclerosis; T1D, type 1 diabetes; UC, ulcerative colitis; DNMT, DNA methyltransferase; MBD, methyl-CpG-binding domain protein.

correlates with a decrease in CD70 promoter methylation in $CD4^+$ T cells compared to healthy controls, as the consequence of the decreased expression of DNMT1.[42] Besides SLE, the reduction of methylation in CD70 promoter also causes overexpression of CD70 in many other autoimmune diseases, such as primary Sjögren's syndrome, systemic sclerosis (SS), and immune thrombocytopenia (ITP).[43–45]

Inerleukin-4 and Interferon-γ

Interferon (IFN)-γ is the typical cytokine of Th1 cells, which is mainly engaged in macrophage activation. Interleukin (IL)-4 is the typical cytokine of Th2 cells, which generally respond by inducing antibody production. The balance between Th1 and Th2 subsets has been implicated in the regulation of many immune responses.[46] $CD4^+$ T cells

treated with 5-azacytidine or procainamide secrete relatively large amounts of IL-4 and IFN-γ,[31,47] implying that DNA methylation participates in T-cell differentiation. Further studies show that the *IFN-γ* and *IL-4* genes are methylated in human naive $CD4^+$ T cells. After stimulation via T-cell receptor (TCR), the IFN-γ promoter becomes hypermethylated in Th2 cells, whereas it is hypomethylated in Th1 cells. Hypermethylation in Th2 cells results in chromatin condensation and exclusion of cAMP response element binding protein (CREB) from IFN-γ promoter.[48,49] Accordingly, the promoter of IL-4 locus becomes specifically demethylated in Th2 cells, but becomes methylated during Th1 differentiation.[50] The hypomethylation of the IL-4 promoter in the pathogenesis of SLE and asthma might be important through upregulating the level of IL-4.[51,52]

FoxP3

The expression of forkhead box P3 (FoxP3) is regulated by epigenetics. It is unmethylated in natural regulatory T cells (nTreg), but heavily methylated in naive $CD4^+$ T cells, activated $CD4^+$ T cells, and peripheral transforming growth factor (TGF)-β–induced Tregs. Thus, FoxP3 DNA demethylation constitutes the most reliable criterion for natural Tregs available at present.[53,54] During T-cell activation in vitro, a DNA demethylation agent, 5-Aza-2'-deoxycytydine, can induce Foxp3 expression in $CD4^+CD25^-Foxp3^-$ cells via altering methylation status of a conserved element in the 5'-untranslated region of the *Foxp3* gene. In addition, the methylation status within the Foxp3 locus in $CD4^+$ T cells is associated with the expression of DNMT1 and DNMT3B, and knocking down the DNMT1 induces FoxP3 expression.[55,56] In latent autoimmune diabetes (LADA) in adultsthe FoxP3 promoter region is hypermethylated in $CD4^+$ T cells from LADA patients compared with the healthy cohort, which may contribute to disease onset and progression of LADA.[56]

Perforin

Perforin is a cytotoxic effector molecule expressed in natural killer cells and a subset of T cells, and it contributes to target cell killing.[57] As a methylation-sensitive gene, the perforin promoter region is hypomethylated in primary $CD8^+$ cells, which express perforin, but is largely methylated in primary $CD4^+$ T cells, which do not.[58,59] It has been reported that perforin is overexpressed in $CD4^+$ T cells from active, but not inactive SLE patients, and this overexpression is related to hypomethylation of the perforin promoter in primary $CD4^+$ T cells.[60,61]

IL-6

IL-6 activates various cell types carrying the membrane bound IL-6 receptor; its signaling plays a pivotal role in controlling the differentiation and activation of T and B lymphocytes. Treatment with 5-aza-2' deoxycytidine induces a high level of IL-6 in MCF-7 (a breast carcinoma cell line) via its gene hypomethylation, which demonstrated that IL-6 expression is influenced by DNA methylation.[62] In SLE patients, the hypomethylation of the CpG islands of the IL-6 promoter occurred in T cells and correlates with symptom severity.[52] In addition, the hypomethylated status of a single CpG in the IL-6 promoter region, such as CpG motif at -74 bp or -1099 bp may augment serum levels of IL-6, implicating a potential role in the pathogenesis of RA and chronic periodontitis.[63,64] In turn, IL-6 may exert many epigenetic changes in cells via increasing expression and activity of DNMTs.[65]

IL-2

IL-2, a key cytokine affecting proliferation and activation of T and B lymphocytes, is also regulated by DNA methylation. During T-cell, B-cell, and macrophage activation, a small region in the promoter-enhancer of IL-2 was demethylated; this demethylated CpG site recruits Oct-1, and induces changes in histone modifications, finally eliciting *IL-2* gene expression.[66,67] Furthermore, the expression of several potential enzymes/co-enzymes in relation to the DNA demethylation pathways such as DNMT1, DNMT3A, and MBD4 seemed to be associated with immune cell activation by regulating the expression of IL-2.[68] Downregulated IL-2 expression is a hallmark of SLE T lymphocytes and is linked to overproduction of the transcription regulatory factor cAMP-responsive element modulator (CREM)α, which binds to a CRE site within the IL-2 promoter and results in epigenetic changes in T lymphocytes through DNMT3A-directed DNA hypermethylation and histone deacetylase-1–directed deacetylation.[69]

CD40 Ligand

CD40 ligand (CD40L), a costimulator expressed on $CD4^+$ T cells, is a response to B-cell activation and immunoglobulin (Ig) class-switch; it is a methylation-sensitive gene that is encoded by X chromosome. Normal $CD4^+$ T cells treated with demethylation agent overexpress CD40L mRNA and induce autologous B-cell activation and plasma cell differentiation. All of these effects can be reversed by anti-CD40L antibody.[70] In patients with SLE, RA, primary biliary cirrhosis, or SS, demethylation of CD40L regulatory elements on the inactive

X chromosome contributes to CD40L overexpression in $CD4^+$ T cells, which may in part explain the female preponderance of autoimmunity.[71–74]

CD5

$CD5^+$ B cells, also called B1 cells, play important roles in the production of high-affinity auto-antibodies; thus they participate in autoimmunity. CD5 has two isoforms: E1A and E1B. E1A is the full-length and transmembrane-directed protein, whereas E1B is a truncated isotype that remains in the cytoplasm.[75] Accordingly, EIA can translocate the Src homology 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) to the vicinity of the B-cell receptor and raise its threshold, thereby limiting the response of autoreactive B cells. In contrast, the truncated variant E1B stays in the cytoplasm, where it can bind to SHP-1 and prevent its effects.[76] The expression of E1B is regulated by DNA methylation. In SLE patients, the promoter for the alternative E1B isoform is demethylated in B cells from patients but not in healthy controls. This finally results in elevated intracellular E1B levels.[77,78]

Others

In addition to the above-mentioned, a large number of methylation-sensitive genes still refer to the pathogenesis of autoimmune diseases (Table 1). Nonetheless, because of the prevalence of CpG islands in genomes, the methylation-sensitive genes related to autoimmune diseases must be more complex than we can imagine. Further research is clearly needed to better understand methylation-sensitive genes in autoimmunity.

DNA METHYLATION AND ITP

Primary ITP is an acquired autoimmune disease characterized by decreased platelet count due to both increased platelet destruction and insufficient platelet production.[111] More than one mechanism could contribute to ITP, including autoreactive B lymphocytes, Th1/Tc1 polarization, T-cell–mediated platelet lysis, abnormal circulating Treg cells, and so on.[112–116] However, until now, the etiology of ITP was incompletely understood. Like in some autoimmune diseases described above, DNA methylation also participates in the pathophysiology of ITP.

Global DNA Methylation Levels in ITP Patients

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) are the donor and receptor of methyl group in the cellular methyltransferase reaction; thus SAM/SAH can be used as an indicator of cellular methylation status. The SAM/SAH ratio in plasma directly correlates with that in lymphocytes, and can reflect cellular methylation levels.[117,118] We measured serum concentrations of SAM and SAH in ITP patients and normal controls using high-performance liquid chromatography, and found that the plasma SAH level was significantly elevated in ITP patients compared with normal controls.[81] In addition, although the SAM/SAH ratio has a tendency to decrease in ITP patients, no significant differences were observed between ITP patients and controls.[81,119] Because SAH has a higher affinity for the methyltransferase active site than SAM,[120] elevated SAH can bind to and inhibit the effect of DNMTs; thus we speculated that lymphocyte DNA hypomethylation may exist in ITP through elevated plasma SAH levels. However, upregulated SAH also can be explained by an increase in DNA methylation, suggesting DNA hypermethylation. Thus further study on the DNA methylation status in ITP patients has been done. Chen et al further assessed global methylation by quantifying the methylcytosine concentration of genomic DNA.[121] The result revealed that $CD4^+$ T cells had a low DNA methylcytosine content in ITP patients, which prompted the conclusion that the hypomethylation pattern occurred in $CD4^+$ T cells of ITP patients. However, the methylation levels seem irrelevant to clinical parameters, including age, sex, clinical course, and platelet counts.[121] Based on these findings, we speculate that the aberrant DNA methylation may take part in the pathogenesis of ITP.

Abnormal Expression of DNA Methylated Enzymes in ITP Patients

The aberrant DNA methylation status is mostly induced by abnormal expression of methylation associated enzymes/co-enzymes. Tao et al found that the mRNA expressions of DNMT3A and DNMT3B were downregulated in peripheral blood mononuclear cells (PBMCs) of ITP patients compared with normal controls. This might be due to the elevated plasma SAH concentrations.[81] Consistent with this result, EL-Shiekh et al found DNMT3A mRNA expression in PBMCs was significantly decreased, while the plasma SAH level was elevated in patients with ITP compared with healthy controls. Thus they speculated that aberrant DNA methylation status, reflected by decreased mRNA expression of DNMT3A and increased plasma SAH level, may engage in the pathogenesis of ITP.[119] However, Ma et al came to a contrary conclusion through the measurement of mRNA expressions of DNMTs in $CD4^+$ T cells from ITP patients.[45] They found that DNMT1, DNMT3A, and DNMT3B were all elevated in $CD4^+$ T cells from ITP patients, and speculated that DNMTs also work as demethyltransferases as well as methyltransferases

in ITP patients.[45] In addition, the detection of mRNA expressions of MBDs showed that the mRNA expression of MBD2 and MBD4 were decreased both in PBMCs and $CD4^+$ T cells in ITP patients compared with those of normal controls,[121] which is different from the result observed in SLE patients.[79] The possible explanation for this discrepancy could be the Th1/Th2 polarization in ITP and the fact that IFN-γ expression was restricted by MBD2.[122]

Methylation-Sensitive Genes in ITP Patients

As a methylation-sensitive gene, aberrant methylation of CD70 is also involved in the etiology of ITP. Firstly, Ma et al detected the expression of CD70 on $CD4^+CD8^+$ T cells and $CD19^+$ B cells and found that both the mRNA and protein expressions of CD70 were both increased in $CD4^+$ T cells from ITP patients, but there was no statistical difference in $CD8^+$ or $CD19^+$ cells between ITP patients and healthy controls.[123] They further detected the DNA methylation indices by polymerase chain reaction (PCR) amplification and high-resolution melting analysis, the result of which demonstrated that the methylation levels of the promoter region of CD70 in ITP were lower than those found in healthy controls. In addition, they found a negative correlation between methylation indices and CD70 mRNA levels in ITP patients and healthy controls. Thus the elevated expression of CD70 may be caused by the hypomethylated promoter region of CD70.[45] This upregulated CD70 can facilitate the survival of T and B lymphocytes, apoptosis of platelets, and secretion of IFN-γ, finally accelerating the progress of ITP.[123]

It is clear that abnormal T-cell immunity including Th1, Th2, Treg, and cytotoxic T lymphocyte (CTL) promotes the development of ITP. As a methylation-sensitive gene, *FoxP3* is the key transcription factor for differentiation of Tregs. Moreover, IFN-γ, IL-4 and perforin are major cytokines secreted by the Th1, Th2, and CTL, respectively. To determine the role of DNA methylation in ITP, Zhao et al[124] investigated the relationship between the mRNA expressions of the *IFN-γ, IL-4, FoxP3,* and *perforin* genes and the methylation status of their respective promoters in PBMCs from ITP patients. However, although the methylation rate of CpGs located at -409 of *perforin* and the whole methylation of the *IFN-γ* gene were higher than those of normal controls, they did not find any negative correlation between the mRNA expression of these genes and the methylation status of their promoters.

Single-Nucleotide Polymorphism and DNA Methylation Defect in ITP Patients

Single-nucleotide polymorphism (SNP) is the most common form of human genetic variation, and may be influence the expression or activity of the protein that was encoded by the mutated gene. This will ultimately result in abnormal biological processes. Several genes in the DNA methylation pathway have been subject to genetic association studies in ITP patients.

Chen et al detected the −149C > T SNP (rs2424913) in the promoter of DNMT3B, but did not find any significantly difference in either genotypes or allelic distribution between ITP patients and normal controls.[125] This result was confirmed by Shaheen et al.[126] In addition, Zhao et al found that there was also no significant difference of another DNMT3B SNP −579G > T (rs1569686) in genotype and allele distribution between the ITP patients and normal controls.[127] Thus it is shown that neither of the two SNPs of DNMT3B can be used as markers to predict the susceptibility to ITP.

MBD4 is different from the other members of the MBD family. It preferentially binds to 5mCpG –TpG mismatches and removes the mismatched thymine or uracil, and thus it is thought to act as a DNA repair enzyme that minimizes mutations at methyl-CpG.[128] Zhao et al[129] investigated the association between MBD4 rs140693 polymorphism and the risk for ITP in a Chinese population. However, there was no significant difference in genotype and allele distribution between the ITP patients and the controls. Similar results were observed between the two groups when stratified by age and disease course, including acute childhood, chronic childhood, acute adult, and chronic adult. In conclusion, MBD4 polymorphism may not be a marker to predict the susceptibility to ITP, at least in the Chinese population.[129]

CONCLUSION

In this review, we have shed light on the important role DNA methylation plays in autoimmune diseases, including ITP, by regulating the expression of methylation-sensitive genes. Thus the identification of a methylation-sensitive gene in ITP will provide a clinical marker of diagnosis, disease progression, and therapy. In addition, for the reversibility of DNA methylation, the site-specific methylated modification may be effective for the treatment of active ITP patients. However, the research about DNA methylation in ITP is still in its preliminary stage, and further experiments are needed to explore its role in ITP.

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